RESEARCH LETTER

Flavobacterium columnare chemotaxis to channel catfish mucus

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Abstract

Flavobacterium columnare is a Gram-negative pathogen of many species of wild and cultured fish. Isolates from diseased channel catfish belong to either genomovar I or II. Genomovar II isolates were found to be more virulent than genomovar I isolates. The objective of the present study was to determine whether differences exist in the chemotactic response of these genomovars to mucus obtained from the skin, gills and intestines of healthy channel catfish using the capillary chemotaxis assay. Mucus from the skin and gill induced a greater chemotactic response by *F. columnare* than mucus from the intestine. Sixty percent of mucus from the skin of individual catfish yielded a positive chemotactic response from *F. columnare*. Finally, skin mucus induced a greater chemotactic response in genomovar II *F. columnare* than in genomovar I *F. columnare* isolates. The data indicate that mucus from channel catfish results in a chemotactic response by *F. columnare*. This positive chemotactic response may be an important first step for *F. columnare* colonization of channel catfish skin or gills. Although the role that chemotaxis plays in the virulence of *F. columnare* is not fully defined, the chemotactic response of genomovar ll isolates suggests that chemotaxis is associated with virulence.

Introduction

The Gram-negative fish pathogen *Flavobacterium columnare* is a causative agent of columnaris disease accompanied by necrotic skin, fin and gill lesions containing yellow-pigmented bacteria, often followed by mortality. This pathogen is responsible for significant economic losses annually in channel catfish, *Ictalurus punctatus*, culture (Wagner *et al.*, 2002). In addition, many other species of wild, cultured and ornamental fish are susceptible (Austin & Austin, 1999). Mucus from the skin and intestine of rainbow trout, *Oncorhynchus mykiss*, has been identified as the chemoattractant for *Vibrio anguillarum* (O'Toole *et al.*, 1999; Larsen *et al.*, 2001). Experimental results indicated that chemotaxis was required for invasion of fish host by *V. anquillarum* (O'Toole *et al.*, 1996). It is also reported that skin mucus was a chemoattractant for *Aeromonas hydrophila* (Hazen *et al.*, 1982). Motility of *F. columnare* and attraction to the skin and gill of catfish leads to the possibility that mucus may be a chemoattractant and may play a role in the virulence of *F. columnare*. The objective of this study was to determine the influence of the *F. columnare* genomovar on the chemotactic response to mucus from skin of channel catfish.

Materials and methods

Flavobacterium columnare isolates and culture conditions

The *F. columnare* isolates and their fish source are shown in Table 1. The first six isolates belong to genomovar II and the last six belong to genomovar I (Shoemaker *et al.*, 2008). Modified Shieh broth was used as a growth medium. The ingredients in modified Shieh were 5.0 g tryptone, 2.0 g yeast extract L^{-1} of distilled water. The pH was adjusted to 7.2 before adding 45.6 μM $CaCl_2 \cdot 2H_2O$, 1.1 μM K_2HPO_4, 1.2 μM $MgSO_4 \cdot 7H_2O$ and 3.6 μM $FeSO_4 \cdot 7H_2O$. The bacteria were aerobically cultured with shaking for 24 h at 27 °C. The bacteria were centrifuged at 2800 ***g*** for 15 min, washed twice in sterile phosphate-buffered saline (PBS), pH 7.2, and resuspended in PBS to 1×10^9 CFU mL^{-1}. The washed bacteria were diluted 1 : 10 in a sterile chemotaxis buffer

(PBS, 0.01 mM EDTA) to a final concentration of 1×10^8 CFU mL^{-1}.

Mucus samples

Mucus was obtained from channel catfish weighing 50–100 g reared at the USDA, ARS Aquatic Animal Health Research Laboratory, Auburn, AL, in flow-through 57-L glass aquaria (aerated water at 25–27 °C and pH 7.2). Fish were anesthetized and mucus samples from the skin, gills and intestines of channel catfish were obtained. Briefly, skin mucus was collected by gently rubbed the lateral surface of the skin using a soft rubber spatula into sterile tubes containing a small amount of PBS (20 μL per tube). Mucus from the gills was collected after dissecting out the gills and carefully rubbing their surface as above. Mucus from the intestine was obtained by cutting out the intestines and washing twice with PBS, followed by rubbing off the mucus into PBS. Gentle rubbing was performed to prevent damage to the surface integrity of the tissues sampled and to avoid contamination with blood or other extraneous products. The mucus samples were centrifuged (6000 ***g*** for 15 min) and the pellet (epithelium cells and cellular debris) was discarded. Mucus samples were culture negative for *F. columnare* on Shieh agar. The protein supernatant concentration was measured by the Bradford method using bovine serum albumin as the standard (Bidlingmeyer *et al.*, 1984). The protein concentration was adjusted with PBS to *c.* 2.1 mg mL^{-1} and then stored at −20 °C. Approximately 2.5–30.0 mg mL^{-1} of protein could be collected from 4.0–4.5 mL^{-1} of mucus. A positive mucus pool was produced from the skin mucus samples of five catfish that triggered a chemotactic response from *F. columnare*. This pool was used to determine whether differences exist in the chemotactic response of these genomovars to mucus from the skin of healthy channel catfish using the capillary chemotaxis assay.

Motility and capillary chemotaxis assay

Measurement of motility was carried out using Shieh plates containing 0.15% agar without a chemoattractant (mucus) modified from Gordillo *et al.* (2007). Briefly, the *F. columnare* isolate was stabbed into the center of the plate and the diameter of motility was determined after a 96-h incubation at 30 °C. The mean motility was determined from six replicate assays of each *F. columnare* isolate tested.

The capillary chemotaxis assay described by Larsen *et al.* (2001) was used. Briefly, mucus samples or chemotactic buffer were dispensed in 5-μL capillary tubes using rubber pipette bulbs [Microcaps (mention of commercial products in this publication does not imply endorsement by the US Department of Agriculture), Drummond, Broomall, PA]. Test or control capillary tubes were suspended in the diluted bacterial suspension dispensed in sterile six-well tissue culture plates (Costar, Corning Inc., Corning, NY). After 90 min, the number of CFU per capillary was determined using a spiral plater (Autoplate 400, Sprial Biotech Inc., Norwood, MA) to dispense the bacteria onto Shieh agar plates. After a 48-h incubation (27 °C), the *F. columnare* colonies were hand counted (Scienceware-Bel-Art Products, Pequannock, NJ). The number of bacteria in the capillaries was calculated as the average from CFU mL^{-1} obtained from duplicate capillary tubes. The mean of two to six independent assays was used to calculate the relative chemotaxis index (RCI). RCI was calculated as the ratio of the bacteria that entered the test mucus capillary to that in the buffer control capillary tube. An RCI of 2 or greater was described as a positive chemotactic response (Mazumder *et al.*, 1999).

Statistics

The motility and chemotaxis data were statistically analyzed using one-way ANOVA, followed by Duncan's multiple range test to determine significant differences between means (SAS, 2000). A 95% confidence interval was considered to be significant.

Results

Motility of *F. columnare* in soft agar

Eleven of the 12 *F. columnare* isolates were shown to be motile in soft agar (Table 1). Generally, the genomovar II isolates were more motile (mean 22 mm diameter) than genomovar I (mean 18 mm diameter). The isolate ATCC-23463 (genomovar I) was significantly less motile and tended to form cell clumps.

Table 1. Isolate, genomovar origin and motility of *Flavobacterium columnare* isolates in 0.15% Shieh agar after 3 days at 30 °C

Isolate	Genomovar	Origin	Motility (mm)
LSU	II	Channel catfish	$21 \pm 2.9^{a,b}$
ALG-00-530	II	Channel catfish	$22 \pm 3.2^{a,b}$
AL-02-36	II	Largemouth bass	$21 \pm 3.6^{a,b}$
ALG-00-515	II	Channel catfish	$21 \pm 4.2^{a,b}$
ALM-05-182	II	Channel catfish	23 ± 2.9^{a}
BGFS-27	II	Channel catfish	24 ± 2.0^{a}
ARS-1	I	Channel catfish	24 ± 1.8^{a}
HS	I	Channel catfish	16 ± 5.4^{c}
MS-02-463	I	Channel catfish	$18 \pm 5.2^{b,c}$
ALM-05-53	I	Channel catfish	$21 \pm 1.1^{a,b}$
ALM-05-140	I	Channel catfish	17 ± 1.2^{c}
ATCC-23463	I	Chinook salmon	13 ± 2.9^{d}

The motility was recorded as the mean diameter and SD of six replicate assays. Groups with different letter superscripts (a, b, c) are significant from each other ($P < 0.05$).

Influence of the tissue origin of mucus

To study the importance of mucus to the motility of *F. columnare*, we first compared mucus obtained from the skin, gill and intestine of channel catfish. The results showed that mucus from the skin and gill was chemoattractant for the genomovar II, *F. columnare* ALG-00-530 (Fig. 1). Mucus from the intestine was less of a chemoattractant for this genomovar II isolate. There was no significant difference in chemotactic response of *F. columnare* to mucus from the skin and gill, but the response was significantly less to mucus from the intestine.

Influence of skin mucus from individual fish

We examined the chemotactic response of the genomovar II, *F. columnare* ALG-00-530, to the skin mucus from 15 catfish. Considerable variation was found for mucus from the skin of individual catfish (Fig. 2). Six of 15 catfish skin mucus samples had chemotactic indexes < 2. A chemotactic index < 2 is not considered to be a positive response. The results showed that variation exists in the attractant ability for *F. columnare* from the skin mucus of individual fish.

Influence of genomovar

We examined the chemotactic response to a positive pool of skin mucus using six genomovar I and six genomvar II isolates of *F. columnare* (Table 2). We showed that genomovar II isolates were significantly more chemotactic than genomovar I isolates. Isolates LSU (genomovar II) and ARS-1 (genomovar I) were the exception. Previously, Shoemaker *et al.* (2008) reported that *F. columnare* of genomovar II was more virulent than those of genomovar I. The genomovar II isolates were reported to cause cumulative mortalities that ranged from 60% to 100%, while

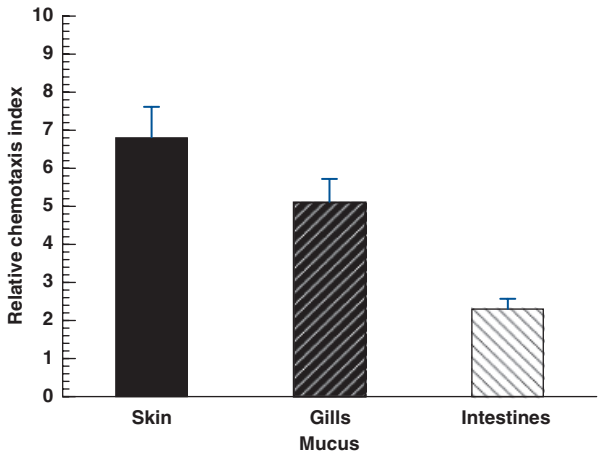

Fig. 1. Chemotactic response of *Flavobacterium columnare* to mucus from skin, gill and intestine of channel catfish. The mean chemotactic response and SD were determined for the ALG-00-530 isolate (genovomar II) using the capillary chemotaxis assay method. The mean of three to six independent assays was used to calculate the RCI. RCI was calculated as the ratio of the bacteria that entered the test mucus capillary to that in the buffer control capillary. An RCI of 2 or greater was described as a positive chemotactic response (Mazumdar *et al.*, 1999). The solid, dark striped and striped bars represent mucus from the skin, gill and intestine, respectively.

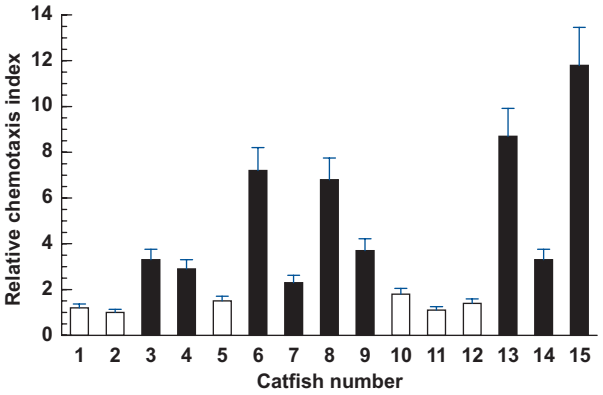

Fig. 2. Chemotactic response of *Flavobacterium columnare* to mucus from skin of 15 channel catfish. The mean chemotactic response and SD were determined for the ALG-00-530 isolate (genovomar II) using the capillary chemotaxis assay method. The mean of three to six independent assays was used to calculate the RCI. RCI was calculated as the ratio of the bacteria that entered the test mucus capillary to that in the buffer control capillary. An RCI of 2 or greater was described as a positive chemotactic response (Mazumdar *et al.*, 1999). The solid and clear bars represent the fish with RCI > 2 and those of < 2, respectively.

Table 2. Comparsion of the *Flavobacterium columnare* RCIs to genomovars

Isolate designation	Control motility CFU per capillary (1×10^4)	Relative chemotaxis index*	*F. columnare* genomovar†
LSU	94	2.6^b	II
ALG-00-530	110	3.0^b	II
AL-02-36	154	2.0^b	II
ALG-00-515	207	2.5^b	II
ALM-05-182	85	5.8^b	II
BGFS-27	74	2.3^b	II
ARS-1	354	1.5^a	I
HS	153	-0.9^a	I
MS-02-463	33	1.4^a	I
ALM-05-53	129	-0.9^a	I
ALM-05-140	185	-0.8^a	I
ATCC-23463	122	-0.9^a	I

*The RCI was calculated as the ratio of the bacteria that enter the test (skin mucus) capillary to that in the control (buffer) capillary. Groups with different letter superscripts (a, b, c) are significantly different from each other ($P < 0.05$).

†Previously reported by Shoemaker *et al.* (2008).

those of genomovar I ranged from 0% to 47%. The results indicated that isolates of genomovar I and II may have different mechanisms of chemotaxis toward skin mucus.

Discussion

The chemotactic response of an *F. columnare* genomovar II isolate to mucus from the skin and gills was demonstrated using the capillary assay (Fig. 1). The chemotactic response to mucus from the intestine was lower. The results suggest *F. columnare* preference for the skin and gill of channel catfish as entry sites in fish host. Bader *et al.* (2003) reported that *F. columnare* cells were attached to the skin and gill mucus as early as 5 min after a bath immersion challenge using a specific PCR. They suggested that the rapid appearance of *F. columnare* on skin tissue may be important in the cutaneous forms of columnaris disease, most often observed in diseased channel catfish.

The chemotactic response of mucus from the skin was found to vary among individual catfish (Fig. 2). Skin mucus from 60% of the fish was chemotactic for the *F. columnare* genomovar II isolate. The variation may be explained by differences in the concentration of the mucus attractant, in the viscosity of the skin mucus samples from individual catfish or the presence of inhibitor(s). Variation in pathogen chemotactic responses to attractants has been reported in rainbow trout (Spanggaard *et al.*, 2000). It is possible that this variation reflects the relative susceptibility of individual fish to columnaris disease. Variation in the susceptibility of catfish challenged by bath immersion with *F. columnare* is well known (Bader *et al.*, 2003; Soto *et al.*, 2007; Shoemaker *et al.*, 2008).

The presence of flagella is also considered to be a pathogen virulence factor (Milton *et al.*, 1996). However, *F. columnare* do not possess flagella, but instead use a gliding motility in chemotactic response (McBride, 2004). The ability of *F. columnare* to adhere to gill tissues (Decostere *et al.*, 1999a, b, 2003) and homogenized whole fry (Shoemaker *et al.*, 2008) has also been associated with virulence. Environmental factors such as water salinity, temperature, stress and nutrition have been reported to influence virulence (Chowdhury & Wakabayashi, 1990; Altinok & Grizzle, 2001; Suomalainen *et al.*, 2005). For example, feed deprivation of channel catfish has been found to increase the susceptibility of channel catfish to columnaris disease (Shoemaker *et al.*, 2003).

Regarding the role of chemotaxis in the virulence of *F. columnare*, deductions can be made from what is known about the pathogenesis of *F. columnare* genomovars. Genomovar II isolates, compared with genomovar I isolates, were demonstrated to be more virulent in catfish (Shoemaker *et al.*, 2008). Genomovar II *F. columnare* isolates appear more attracted to mucus from the skin of channel catfish (Table 2). Thus, we proposed that the presence of chemoattrants in the host mucus from the skin and gills has implications for a relationship between chemotaxis and *F. columnare* virulence.

The attractant(s) in mucus from the skin of channel catfish was not identified. The chemotactic response of *V. anguillarum* to fish intestinal mucus was reported to be mediated by a combination of multiple mucus components (O'Toole *et al.*, 1999). Possible mucus attractants may include amino acids, peptides, sugars, simple or complex carbohydrates, glycopeptides, glycolipids and other chemicals. Skin mucus is known to comprise a number of immune components such as lysozyme, immunoglobulin, complement, carbonic anhydrase, lectins, crinotoxins, calmodulin, C-reactive protein, proteolytic enzymes and antimicrobial peptides (Alexander & Ingram, 1992). Because chemotaxis is the movement of bacterial cells toward a chemical gradient of a substance that it recognizes, the skin mucus chemoattractant(s) should be able to interact with the bacterial surface receptors and thereby stimulate cell movement (Herrmann & Burman, 1983). We speculated that the chemical nature of the skin mucus attractant is a lectin-like substance that may bind *F. columnare* carbohydrate receptors on its capsule. Alternatively, the bacteria may be moving in response to a nutrient gradient. Staroscik & Nelson (2008) suggest that *F. columnare* was able to utilize skin mucus from Atlantic salmon, *Salmo salar*, as a growth substrate.

In conclusion, the results suggest that mucus from the skin and gills of channel catfish is a chemoattractant. Analysis of chemotactic response to skin mucus from individual catfish revealed that the mucus attractant was not always demonstrable. Also, the results indicated that the susceptibility of previously uninfected channel catfish to columnaris is dependent on the host mucus attractant and the *F. columnare* genomovars.

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